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Physiological extracellular electrical signals guide and orient the polarity of gut epithelial cells

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Apical-basal polarity in epithelial cells is a fundamental process in the morphogenesis of many tissues. But how epithelial cells become oriented with functionally specialized luminal and serosal facing membranes is not understood fully. Cell-cell and cell-substrate contacts induce the asymmetric distribution of Na^+/K^+-ATPase pumps on basal membrane and are essential for apical-basal polarity formation. Inhibition of the Na^+/K^+-ATPase pump abolished apical formation completely. But it is unclear how this pump regulated the apical polarity. We discovered that the transepithelial potential difference (TEP) which is dependent on the basal Na^+/K^+-ATPase distribution acts as an essential coordinating signal for apical membrane formation through Ror2/ERK1/2/LKB1 signaling. A similar concept applies to all other ion-transporting epithelial and endothelial tissues and this raises the possibility of regulating the TEP as a therapeutic intervention for disorders in which epithelial function is compromised by faulty electrical signaling.

Keywords: apical-basal polarity, cell polarization, extracellular electrical signal, Na^+/K^+-ATPase, transepithelial potential difference

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Commentary on 'Polarizing intestinal epithelial cells electrically through Ror2' *J Cell Sci.* 2014, 127:3233

The formation of epithelial layers with apical-basal polarity is a fundamental process in the morphogenesis of many tissues including intestine, lung, skin and kidney. The polarity of epithelial cells involves apical and basolateral regions, with different molecular components and structure. Apical polarity proteins including a transmembrane protein (Crumbs), a lipid phosphatase (PTEN), a small GTPase (Cdc42), FERM (Band 4.1, Ezrin, Radixin and Moesin) domain proteins, and several adaptor or scaffolding proteins (Bazooka/Par3, Par6, Stardust, Patj) form a dynamic cooperative network that is engaged in regulation with basolateral polarity factors to set up the epithelial apical-basal axis.[1] In three dimensional cultures of Caco-2 cells, ouabain as an inhibitor of basal Na^+/K^+-ATPase pump inhibits lumen expansion completely.[2] In addition, inhibition of Na^+/K^+-ATPase and its downstream RhoA GTPase prevented the formation of tight junctions and desmosomes and the cells remained nonpolarized.[3] However, how the basal Na^+/K^+-ATPase pump regulates the epithelial polarity is uncertain. Our recent discovery presents a new and additional interpretation, namely that the transepithelial potential difference (TEP) based on Na^+/K^+-ATPase expression on the basal membrane acts as a bioelectrical signal to regulate apical membrane formation.[4]

The TEP is an extracellular bioelectrical signal which has been shown to provide a directional signal for cell migration, wound healing and neurite growth.[5] The TEP is an inherent property of transporting epithelia and arises from spatial variations in the function of ion pumps, channels, or leak conductance across individual cells, and therefore collectively across a layer of cells.[5] An asymmetric distribution of ion pumps and channels, e.g. Na^+/K^+-ATPase on basal membranes, is one of the important features of apical-basal polarity in most epithelia. Recently, we discovered that mimicking the TEP imposed a polarity on single cells and epithelial sheets which suggests a functional role for the TEP as an extracellular signal that activates ERK1/2 and LKB1 in establishing apical-basal polarity in intestinal epithelium. Here, we will focus on insights into how the TEP as a guidance signal may link and coordinate the

interactions in directed cell migration, wound healing and polarity of epithelia.

TEP- Guidance Cues in Cell Migration, Wound Healing and Neurite Growth

Epithelial cells generate electrical gradients within conductive extracellular spaces by polarized ions transport which separates ions between the apical and basal domains. The voltage gradient across the intestinal epithelium (TEP) is lumen side negative. This voltage gradient depends on 1) activation of selective ion channels, transporters and pumps restricted to the apical or basolateral membranes that establish ionic gradients across the epithelium and 2) tight junctions between neighboring cells in the intact epithelium which by electrically "sealing" the cells to each other limit paracellular movement of ions[5,6] (**Fig. 1A**). For instance, the basal localization of the Na^+/K^+-ATPase pumps drives sodium ions to the basal side (moving 3 Na^+ out and 2 K^+ into cells), thus generating a voltage drop between the basal and the luminal side.[7] Na^+ influx through apical Na^+-channels maintains homeostatic levels of cytosolic Na^+, while Cl^- efflux from the apical membrane also may help to generate the internally positive TEP.[8]

Across human intestine therefore, there is a TEP of -25 ± 7 mV, lumen negative.[9] This is the equivalent of a direct current (DC) electric field (EF) across the epithelial layer of around 500 mV/mm, since the epithelium of human intestine is about 50μM thick.[9] The functional roles of the TEP in intestine are not fully understood. In other epithelia, damage to the high-resistance epithelial structure leads to short circuiting of the TEP and the flow of extracellular current out at a lesion site, e.g., in skin and cornea.[5,10] The center of the epithelial wound is electrically negative with respect to the undamaged regions surrounding the wound edge.[10] An applied EF signaling through PI3K/PTEN predominated over coexisting chemical gradients in controlling wound healing in a monolayer scratch model.[11,12] Fibroblasts from embryonic quail migrated directly toward the cathode in an applied EF of 1–10 mV/mm field strength.[13,14] In brain, we measured an extracellular bioelectrical signal of 3–5mV/mm between the subventricular zone (SVZ) and the olfactory bulb (OB).[15] Mimicking this with an applied EF induced directed/chain migration of neuroblasts cathodally,[16] while astrocytes and Schwann cells show oriented growth in EFs as low as 3 mV/mm and Schwann cells migrate rapidly anodally.[17,18] Defects in neuronal migration may lead to important diseases including lissencephaly, epilepsy and mental retardation.[19-21] An applied EF also stimulates neuronal cells to differentiate into neurons by sending out neurites.[22] It is as though the cells need an external directional signal in order to begin the highly polarized process of neurite formation. The fields of negative polarity attract the growth cone, whereas fields of positive polarity deflect the

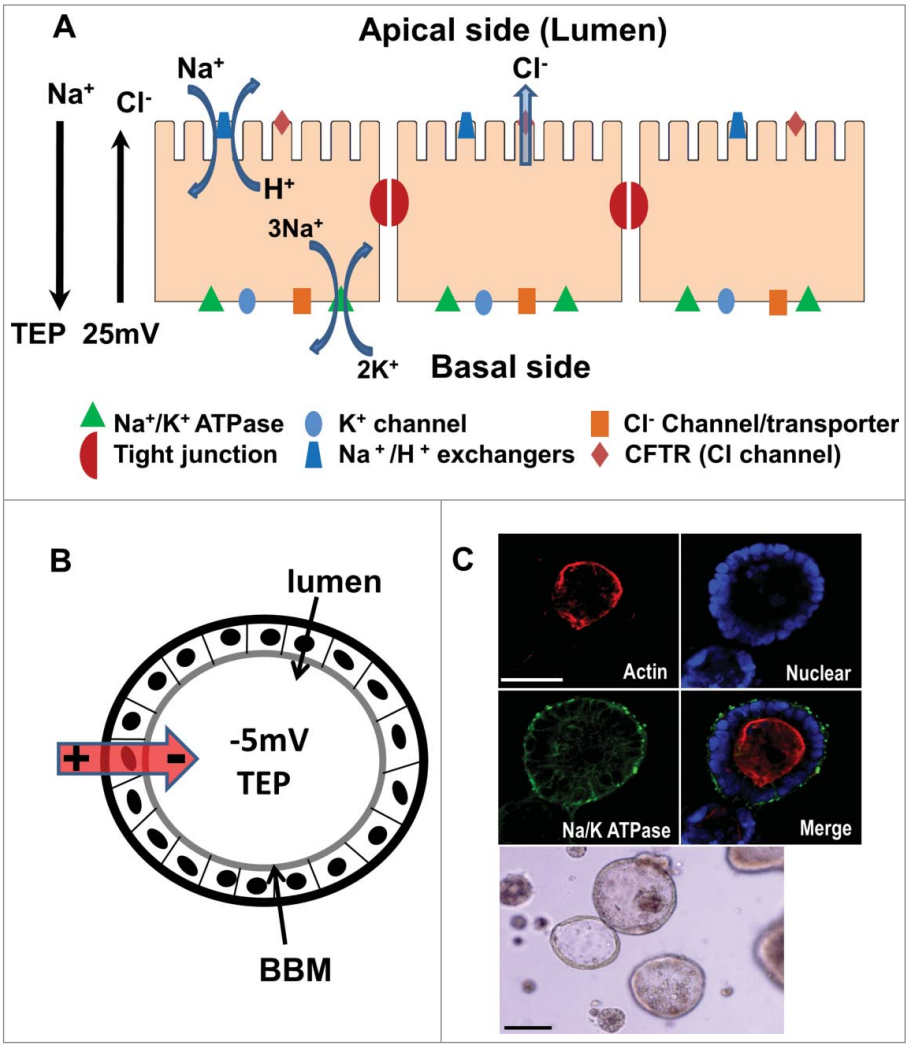

Figure 1. Illustration of the generation of the transepithelial potential differences (TEP) and distribution of Na^+/K^+-ATPase in enterocytes. (**A**) Polarized localization of specific ion channels pumps and transporters establish the TEP. Na^+/K^+-ATPase which is located on the basal membrane uses energy from ATP hydrolysis to drive Na^+ extrusion and K^+ uptake (3:2 stoichiometry), both moving against their electrochemical gradients. The CFTR chloride channel is localized apically. The opening of CFTR enables an inward flux of Cl^- into the lumen. The consequent separation of charge across the epithelium results in a measurable TEP difference of 25 mV in human. (**B**). The intestinal epithelium supports a TEP of −5 mV (lumen negative relative to the serosal side) in human intestinal epithelial cells C2BBe1 cyst culture. (**C**). C2BBe1 cells were cultured on Matrigel for 7 d. Single confocal sections through the middle of cysts were stained for Na^+/K^+ ATPase (green), Actin (red), DNA (blue) and overlay. Phase-contrast image before staining is shown. Bar = 75 μm.

growth cone away.[23] These findings suggest that extracellular EFs act as guidance cues in cell migration, wound healing and neuronal development/regeneration.

The TEP is an Orientation Signal that Coordinates Apical Polarity

In epithelia, the formation of adhesive contacts between cells and between cell–ECM are among the earliest steps in forming a functional tissue.[24] Shortly thereafter, the expression of basolateral membrane molecules of epithelium (e.g. Na^+/K^+-ATPase) is triggered and generates the TEP (**Fig. 1B, C**). The TEP therefore is a tissue level signal generated soon after tight junction formation and basal polarization. Inhibition of Na^+/K^+-ATPase reduced significantly the TEP[25] and abolished apical (lumen) actin polarity in 3D cyst culture of Caco-2 cells.[2] This suggested that the TEP as a basal signal could be a signal to mediate the apical polarization in gut. We reported recently that the natural extracellular bioelectrical signal across the intestinal epithelium (TEP) encodes epigenetically the information required for cell and tissue level polarization.[4] We selected the LS174T-W4 cell line (supplied by Professor Clevers's lab, Hubrecht Institute, Netherlands) as a model of epithelial polarity in which the polarity orientation could be induced by an electrical signal. LS174T-W4 cells have some important features such as: (1) Complete polarization: LS174T-W4 cells develop an inducible expression of LKB1 and can form a brush border-like structure in single cell culture;[26,27] (2). Lack of electrotaxis (no directed movement in an applied electric field): therefore making it impossible that polarized accumulation and reorientation of some polarity markers, e.g., ezrin, actin and CD66 could result from a directed motility response to the applied EF; (3). No cell-cell tight junctions: thus excluding some factors which are associated with cell-cell connection as causal in polarity formation. By using this model, we identified that an applied EF (**Fig. 2**) which mimics the TEP could play a role to set-up orientation in apical-basal polarity of intestinal epithelial cells (**Fig. 3A**). Furthermore in transwell cultures, C2BBe1 cells established an electrical potential difference (TEP) across themselves by transporting ions that generate concentration gradients[5] (**Fig. 3C and D**). Inhibition of ion transportation with ouabain and digoxin significantly reduced the TEP and resulted in formation of the brush border membrane (BBM) being inhibited (**Fig. 3D and E**). These data support the notion that the TEP may serve as a coordinating signal in intestinal epithelial cell polarity, specifically by imposing apico-basal polarity to ensure the correct apical localization of the BBM in enterocytes.

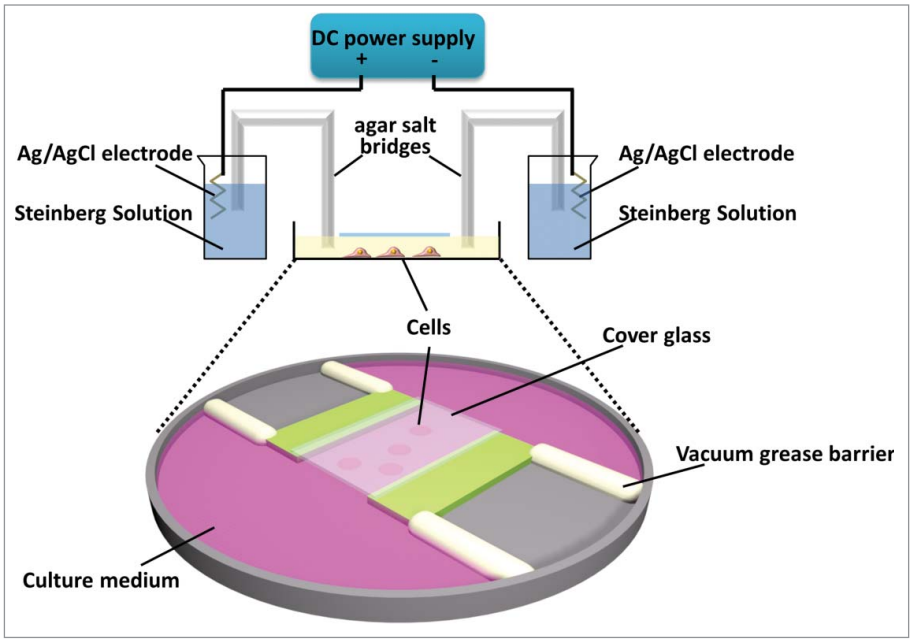

Figure 2. The device for applying electric fields to cells to mimic the TEP. Cells were exposed to a DC electric field applied across the central chamber. The cells were cultured under a cover slide and 2 agar salt bridges were used to connect the culture medium with the power supply, so as to eliminate toxic electrode products. The EF vector was parallel to the long axis of the chamber which was delineated by the two green strips of cover glass. This figure is derived from Cao et al, 2014.[4]

Molecular Mechanisms Underpinning TEP-Induced Apical Polarity

Some secreted proteins e.g. Wnt proteins act as extracellular mechanisms to determine neuronal polarity along the anterior–posterior body axis.[28] Wnts as a

Table 1. Applied EF promote secretion of cytokines and other molecules

Name	EF	Frequency	Model	Reference
VEGF	200 mV/mm	DC	HUVEC	Zhao et al, 2004
Insulin and glucagon	50 V	5–20Hz	Normal and diabetic rats	Adeghate et al, 2001
Neurotrophic factor (BDNF)	15 V	50–100Hz	Hippocampal neurons	Gärtner and Staiger 2001
ATP	200 V/mm	DC	Chromaffin cells	Rojas et al, 1985
ATP	750 mV/mm	DC	Multicellular tumor spheroids	Heinrich et al, 2002
VEGF and IL-8	200 mV/mm	DC	Human endothelial cells	Bai et al, 2011

group of secreted, lipid-modified glycoproteins trigger intracellular responses through canonical or noncanonical pathways.[29] The Wnt5a protein is expressed in normal colon epithelium and the highest level of expression is at the base of the intestinal crypts.[30] Wnt5a interacts with the orphan tyrosine kinase receptor Ror2 to control planar cell polarity in epithelia. An applied EF can increase the secretion of proteins and other compounds e.g., VEGF, ATP and etc (**Table 1**).[31,32] We recently showed that an applied EF effectively activated ERK1/2 and LKB1 in LS174T-W4 cells (**Fig. 3B**). Interruption of Ror2 and ERK1/2 significantly inhibited the activation of LKB1 which has been identified as a key molecule in inducing complete polarity in intestinal epithelial cells.[4,26,33] This raises the possibility that the TEP could regulate the secretion of Wnt5a into the lumenal side of intestine and there activate ERK1/2 and LKB1 through the Ror2 receptor.[4]

The Golgi complex in polarized epithelial cells is typically oriented toward the apical plasma membrane domain. The membrane proteins which are localized on the apical and basolateral aspects of epithelial cells are synthesized in the endoplasmic reticulum, transferred to the Golgi apparatus and segregated into different post-Golgi transport intermediates (PGTIs) for export to the cell surface.[34–36] We have shown that the polarization of the Golgi apparatus in CHO cells is determined by an applied physiological EF.[37,38] We found that the applied physiological EF induced the Golgi to redistribute to the cathode side/leading edge during directed migration of CHO cells and regulated the direction of this migration (**Fig. 4**). This suggests that the TEP in the lumen of gut may redistribute and organize the Golgi apparatus toward the lumen/apical side.[37] We shall investigate further whether the TEP is necessary in the establishment of gut epithelial polarity through Wnt5a/Ror2 signaling and Golgi polarity *in vivo*.

In addition, the Par3/Par6/aPKC complex is a master regulator of polarity.[39] PTEN also is associated with the apical membrane in the 3D structure of mammalian cells.[40] The physiological EFs can regulate the activation or expression of cell polarity-related proteins including PKC, GSK-3β and PTEN.[11,37,41] Cdc42 is needed for fusion of transport vesicles to the apical surface and creation of the lumen by controlling spindle orientation during

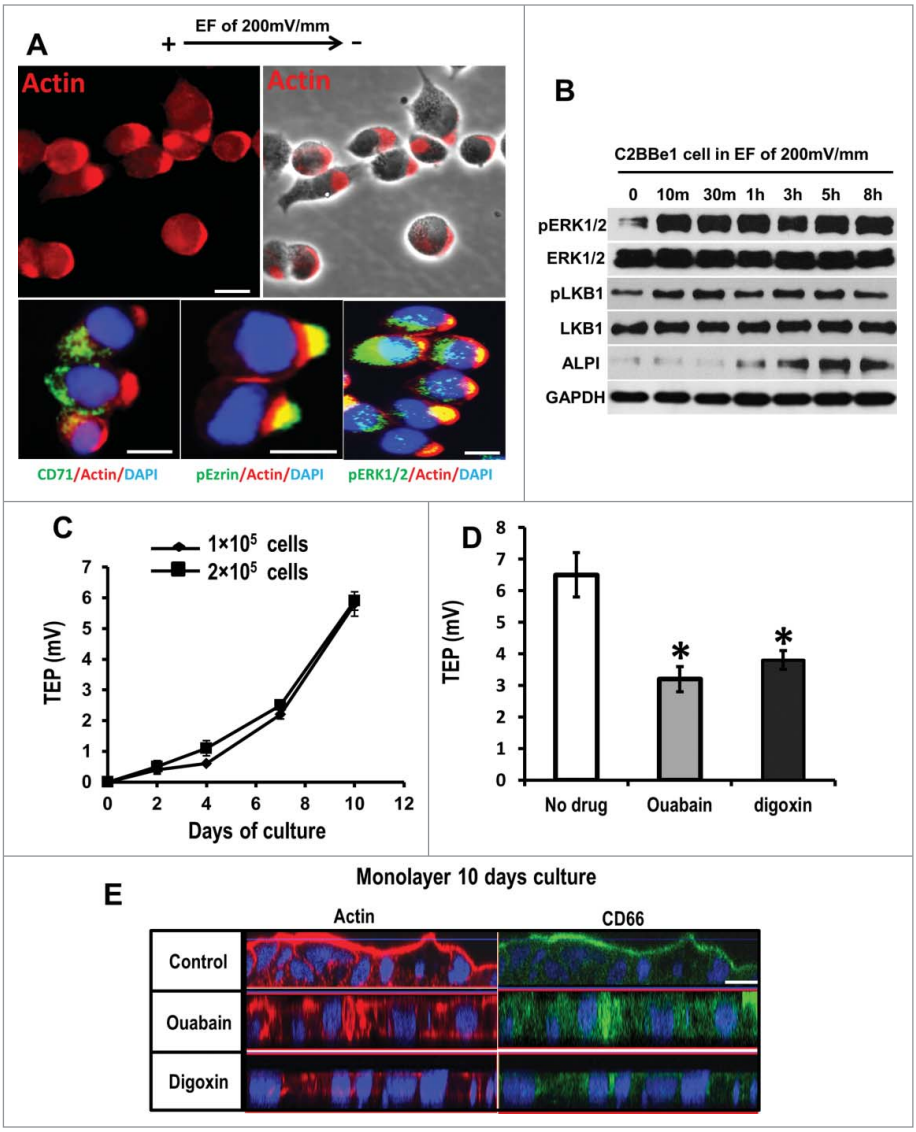

Figure 3. The role of the electric field in the apical-basal polarity of intestinal epithelial cells. (**A**) LS174T-W4 cells were treated with the applied electric field in a specific chamber which has been described in **Figure 2** and Dox for 24 hours. Cells were fixed and stained with phalloidin (F-actin label indicative of apical membrane), DAPI and the basal membrane marker CD71, phosphorylated Ezrin and ERK1/2, respectively. Upper images: EF stimulation causes a polarization of F-actin on the cathodal side of the cells (at right). Lower images: polarized actin labeling faced the cathode and CD71 was localized selectively at the anode side. Actin and phosphorylated Ezrin or ERK1/2 colocalized and polarized toward the cathodal side of cells. Bar = 10μm. (**B**). The time course of EF-induced activation of pERK and pLKB1 and enhanced ALP1 expression in C2BBe1 cells. The expression of total ERK1/2 and LKB1 were not affected by EF. (**C**) C2BBe1 cells form tight junction complexes which allow the generation of a steady time-dependent increase of transepithelial potential difference (TEP) in insert monolayer cultures. The TEP keeps increasing over 10 d. (**D**). The TEP across the cyst wall was reduced significantly by Na^+/K^+-ATPase inhibitors (or TEP inhibitors), ouabain or digoxin. (**E**) Monolayer of C2BBe1 cells were cultured on a glass slides for 10 d. Confocal images (z-axis scanning) showed that the pronounced apical membrane architecture of C2BBe1 monolayers (upper, apical staining with phalloidin-TRITC and CD66) was disrupted by suppression of the TEP using the inhibitors ouabain and digoxin (lower). Bar = 20μm. GAPDH was loading control for western blot. Modified from figures in Cao et al, 2014.[4]

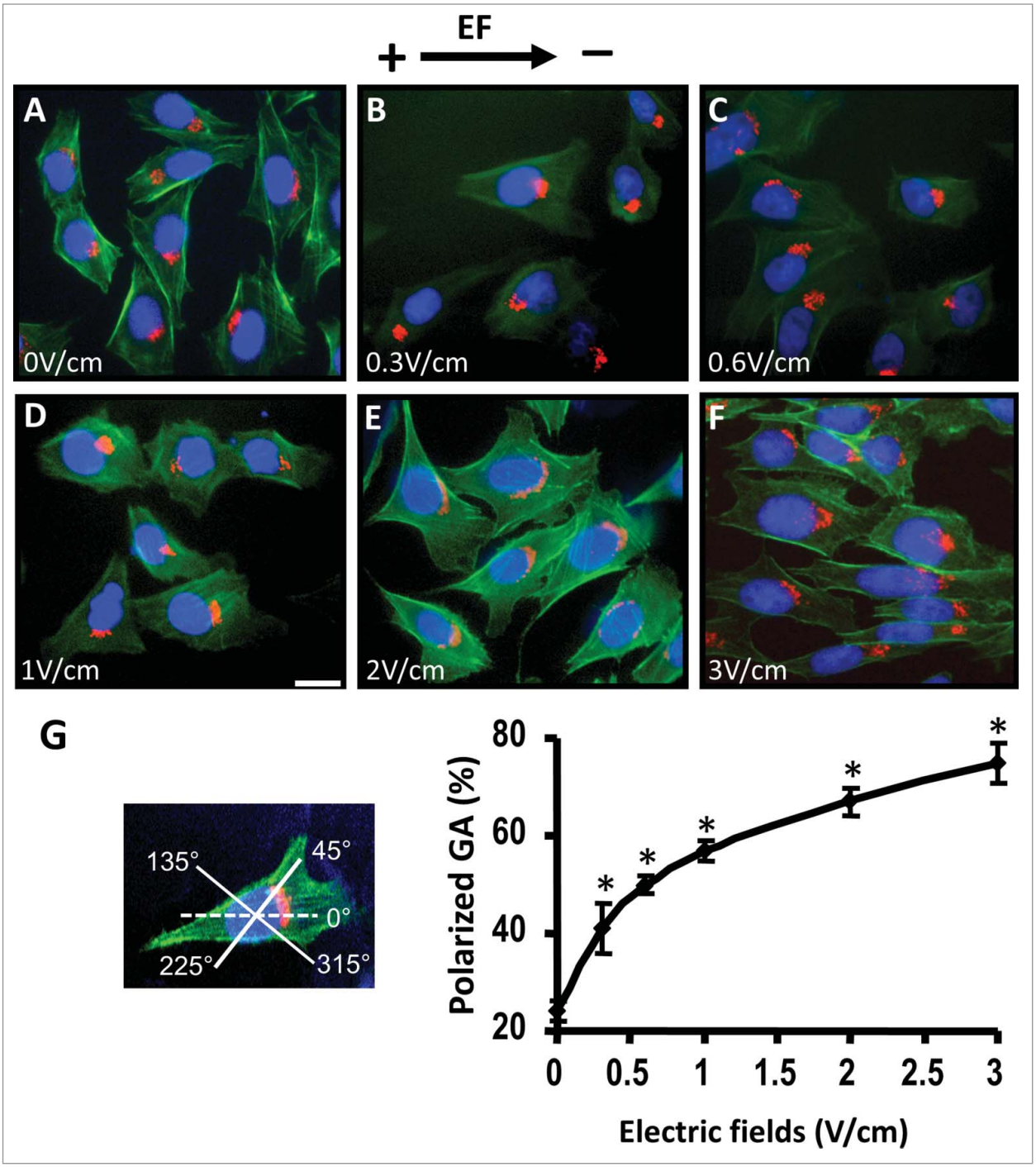

Figure 4. EFs of physiological strength directed Golgi apparatus (GA) polarization in CHO cells. (**A–F**) EFs were applied to CHO cells for 3 hours and then were fixed and triple-labeled with GM130 antibody (GA marker, red), FITC-phalloidin (F-actin, green) and DAPI (blue). Fluorescent images showed that the EF strongly polarized the Golgi toward the cathode/leading edge in voltage dependent manner. (**G**). GA polarization was quantified as the percentage of GA polarized into the quadrant between 45 and 315 degrees in the field direction as shown. *$P < 0.01$ compared to no EFs control. Bar = 20 μm. The figure is derived from Cao et al, 2014.[37]

cell division.[2,40,42] An applied EF affected cell polarity and determined the directional growth in yeast cells through small GTPase cdc42p.[43] The applied EF also directed growth cone cathodal steering through Cdc42.[44] These data all suggest that extracellular bioelectrical signals may contribute to the intracellular molecular mechanisms in the apical polarity formation.

In summary, correct polarization of enterocytes is critical for apical BBM formation and the directional absorptive and secretory functions of the gut. The physiological electrical signal in the extracellular

space acts as a robust, global signal to induce enterocyte polarization and apical BBM formation. New insights in apical-basal polarity may provide new avenues of research in epithelial disorders with a known involvement of barrier disruption, e.g. severe malnutrition and persistent osmotic diarrhea. The absence of apical-basal polarity in epithelia may lead also to cancer and polycystic kidney disease (Fischer et al., 2006; Royer and Lu, 2011). Regeneration of the BBM of intestine using an applied EF or as yet unidentified regulators of bioelectrical signal could prove to be an interesting field and raises the possibility of regulating the TEP as a therapeutic intervention to treat pathologies in a number of other epithelia.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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